KUZ, A Novel Family of Metalloproteases

Inventors: Gerald M. Rubin, Duojia Pan, Jenny Rooke, Reza Yavari and Tian Xu
Assignees: The Regents of the University of California and Yale University

This application is a divisional of USSN 08/937,931, filed August 27, 1997, which claims priority to US Provisional Application Serial No. 60/019,390 filed August 29, 1996 and to US Provisional Application Serial No. 60/053,476 filed July 23, 1997, each of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The field of the invention is a novel family of proteins and genes involved in development.

BACKGROUND OF THE INVENTION

Cell-cell interactions play an important role in regulating cell fate decisions and pattern formation during the development of multicellular organisms. One of the evolutionarily conserved pathways that plays a central role in local cell interactions is mediated by the transmembrane receptors encoded by the *Notch* (*N*) gene of Drosophila, the *lin-12* and *glp-1* genes of C. elegans, and their vertebrate homologs (reviewed in Artavanis-Tsakonas, S., et al. (1995) Notch Signaling. Science *268*, 225-232). collectively hereinafter referred to as NOTCH receptors. Several lines of evidence suggest that the proteolytic processing of NOTCH receptors is mportant for their function. For example, in addition to the full length proteins, antibodies against the intracellular domains of NOTCH receptors have detected C- terminal fragments of 100-120 kd (hereafter referred to as 100 kd fragments); see e.g. Fehon, R. G., et al. (1990). Cell *61*, 523-534; Crittenden, S. L., et al. (1994). Development *120*, 2901-2911; Aster, J., et al. (1994) Cold Spring Harbor Symp. Quant. Biol. *59*, 125-136; Zagouras, P., et al.(1995). Proc. Natl. Acad. Sci. USA *92*, 6414-6418; and Kopan, R., et al. (1996). Proc. Natl. Acad. Sci. USA *93*, 1683-1688. However, the mechanism(s) of NOTCH activation have been hitherto largely unknown.

During neurogenesis, a single neural precursor is singled out from a group of equivalent cells through a lateral inhibition process in which the emerging neural precursor

cell prevents its neighbors from taking on the same fate (reviewed in Simpson, P. (1990). Development *109*, 509-519). Genetic studies in Drosophila have implicated a group of "neurogenic genes" including *N* in lateral inhibition. Loss-of-function mutations in any of the neurogenic genes result in hypertrophy of neural cells at the expense of epidermis (reviewed in Campos-Ortega, J. A. (1993) In: *The Development of Drosophila melanogaster* M. Bate and A. Martinez-Arias, eds. pp. 1091-1129. Cold Spring Harbor Press.). We disclose herein a new neurogenic gene family, *kuzbanian* (*kuz*) (Rooke, J., Pan, D. J., Xu, T. and Rubin, G. M. (1996). Science *273*, 1227-1231). Members of the disclosed KUZ family of proteins are shown to belong to the recently defined ADAM family of transmembrane proteins, members of which contain both a disintegrin and metalloprotease domain (reviewed in Wolfsberg, T. G., et al. (1995). J. Cell Biol. *131*, 275-278, see also Blobel, C. P., et al. (1992). Nature *356*, 248-252, 1992; Yagami-Hiromasa, T., et al. (1995). Nature *377*, 652-656; Black, R. A., et al. (1997). Nature *385*, 729-733, 1997; and Moss, M. L., et al. (1997). Nature *385*, 733-736).

We further disclose herein various engineered mutant forms of native KUZ proteins and their activities. We show that mutant KUZ proteins lacking protease activity interfere with endogenous KUZ activity and function as dominant negatives (indicating that the protease activity of native KUZ is essential to its biological functions) and that dominant negatives can perturb lateral inhibition during neurogenesis and result in the overproduction of primary neurons. We also show that proteolytic processing of NOTCH in embryos to generate the 100 kd species fails to occur in the *kuz* mutant embryo and expression of dominant negatives in imaginal discs or tissue culture cells blocks NOTCH processing (indicating that the primary NOTCH translation product is proteolytically cleaved by native KUZ proteins as part of the normal biosynthesis of a functional NOTCH receptor).

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to isolated KUZ polypeptides, related nucleic acids, polypeptide domains thereof having KUZ-specific structure and activity and modulators of KUZ function, particularly Notch protease activity. KUZ polypeptides, nucleic acids and modulators thereof regulate Notch signal transduction pathways and hence provide important regulators of cell function. The polypeptides may be produced recombinantly from transformed host cells from the subject KUZ polypeptide

encoding nucleic acids or purified from mammalian cells. The invention provides isolated KUZ hybridization probes and primers capable of specifically hybridizing with the disclosed KUZ genes, KUZ-specific binding agents such as specific antibodies, and methods of making and using the subject compositions in diagnosis (e.g. genetic hybridization screens for KUZ transcripts), therapy (e.g. KUZ protease inhibitors to modulate Notch signal transduction) and in the biopharmaceutical industry (e.g. as immunogens, reagents for isolating additional natural *kuz* alleles, reagents for screening bio/chemical libraries for ligands and lead and/or pharmacologically active agents, etc.).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A). Sequence alignment of predicted KUZ proteins from Drosophila (DKUZ), mouse (MKUZ) and Xenopus (XKUZ). The full length amino acid sequence of MKUZ was deduced from the nucleotide sequence of two overlapping cDNA clones. Partial amino acid sequence of XKUZ was deduced from the nucleotide sequence of a PCR product that includes parts of the disintegrin and Cys-rich domains. The alignments were produced using Geneworks software (IntelliGenetics). Residues identical among two species are highlighted. Predicted functional domains are indicated. Amino acid sequences from which degenerate PCR primers were designed are indicated with arrows. Orthologs of *kuz* are also present in C. elegans (GenBank accession nos. D68061 and M79534), rat (Z48444), bovine (Z21961) and human (Z48579).

Figure 1(B). Summary of constructs used in this study and their overexpression phenotypes. Different domains are indicated by shadings. Asterisks indicate where point mutations were introduced. Constructs 1-9 are based on DKUZ, while MKUZDN is based on MKUZ. Abbreviations: ++, strong phenotype; +, weak phenotype; 0, no phenotype.

Figure 1(C). Schematic diagram of DKUZ, MKUZ and XKUZ. The percentages given refer to sequence identity in the indicated domains between MKUZ and either DKUZ or XKUZ.

Figure 2 shows a schematic of how KUZ protease can process NOTCH on the extracellular domain to generate an N- terminal extracellular fragment and the C-terminal 100 kd fragment containing the transmembrane and the cytoplasmic domain. These two fragments

may remain tethered together to function as a competent NOTCH receptor, analogous to the maturation of the SEVENLESS receptor (Simon et al., 1989).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides isolated KUZ polypeptides, isolated from a wide variety of sources including *Drosophila*, human, mouse and *Xenopus*, as well as allelic variants, naturally occurring and altered secreted forms, deletion mutants having KUZ-specific sequence and/or bioactivity and mutants comprising conservative amino acid substitutions. SEQ ID NOS:1, 3, 5, 7 and 9 depict exemplary natural cDNAs encoding *Drosphila*, human transmembrane, human soluble (lacking a transmembrane domain), mouse and *Xenopus* members, respectively, of the disclosed KUZ family. SEQ ID NOS: 2, 4, 6, 8 and 10 depict the corresponding encoded full-length KUZ proteins. Methods used to isolate additional members of the *kuz* family are described below and in the Examples.

Preferred translates/deletion mutants comprise at least a 10, preferably at least a 15, more preferably at least a 20 residue domain of at least one of SEQ ID NOS:2, 4, 6, 8 and 10. In particular, KUZ derivatives can be made by altering *KUZ* sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *kuz* gene may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of *kuz* genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the KUZ derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a KUZ protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Conservative substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine proline, phenylalanine,

tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a KUZ protein consisting of at least 10 (continuous) amino acids of the KUZ protein is provided. In other embodiments, the fragment consists of at least 15 or 20 or 50 amino acids of the KUZ protein. In specific embodiments, such fragments are not larger than 35, 100 or 200 amino acids. Derivatives or analogs of KUZ include but are not limited to those peptides which are substantially homologous to a KUZ protein or fragments thereof. (*e.g.*, at least 30%, 50%, 70%, or 90% identity over an amino acid sequence of identical size--*e.g.*, comprising a domain) or whose encoding nucleic acid is capable of hybridizing to a coding *KUZ* sequence.

The subject domains provide KUZ domain specific activity or function, such as KUZ-specific protease or protease inhibitory activity, disintegrin or disintegrin inhibitory activity, ligand/antibody binding or binding inhibitory, immunogenicity, etc.; see, e.g. domains identified in Fig. 1A-C. Preferred domains cleave a NOTCH protein. KUZ-specific activity or function may be determined by convenient *in vitro,* cell-based, or *in vivo* assays: e.g. *in vitro* binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of an KUZ polypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as an KUZ substrate, a KUZ regulating protein or other regulator that directly modulates KUZ activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or an KUZ specific agent such as those identified in screening assays such as described below. KUZ-binding specificity may assayed by protease activity or binding equilibrium constants (usually at least about $10^7 M^{-1}$, preferably at least about $10^8 M^{-1}$, more preferably at least about $10^9 M^{-1}$), by the ability of the subject polypeptide to function as negative mutants in KUZ-expressing cells, to elicit KUZ specific antibody in a heterologous host (e.g a rodent or rabbit), etc. The KUZ binding specificity of preferred KUZ polypeptides necessarily distinguishes that of the bovine protein of Howard, L., et al. (1996). Biochem. J. *317*, 45-50.

The claimed KUZ polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The KUZ polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably human cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art. Material and methods for the expression of heterologous recombinant proteins in bacterial cells (e.g. E. coli), yeast (e.g. S. Cerevisiae), animal cells (e.g. CHO, 3T3, BHK, baculovirus-compatible insect cells, etc.). The KUZ polypeptides and/or domains thereof may be provided uncomplexed with other protein, complexed in a wide variety of non-covalent associations and binding complexes, complexed covalently with other KUZ or non-KUZ peptide sequences (homo or hetero-chimeric proteins), etc.

The invention provides binding agents specific to the claimed KUZ polypeptides, including substrates, agonists, antagonists, natural intracellular binding targets, etc., methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins. Novel KUZ-specific binding agents include KUZ-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate KUZ function, e.g. KUZ-dependent proteolytic processing. For example, a wide variety of inhibitors of KUZ Notch

protease activity may be used to regulate signal transduction involving Notch. Metalloprotease and disintegrin inhibitors and methods for designing such inhibitors are well known in the art, e.g. Matrisian, L. TIG, 6:(1990), Hooper, N. FEBS Let. 354:1-6 (1994), Haas et al., Cur. Op. Cell Bio. 6:656-662 (1994), etc. Exemplary inhibitors include known classes of metalloprotease inhibitors, KUZ-derived peptide inhibitors, esp. dominant negative deletion mutants, etc. KUZ specificity and activity are readily quantified in high throughput protease assays using panels of proteases.

Accordingly, the invention provides methods for modulating signal transduction involving Notch in a cell comprising the step of modulating KUZ protease activity, e.g. by contacting the cell with a protéase inhibitor. The cell may reside in culture or in situ, i.e. within the natural host. For use in methods applied to cells in situ, the compositions frequently further comprise a physiologically acceptable excipient and/or other pharmaceutically active agent to form pharmaceutically acceptable compositions. Hence, the invention provides administratively convenient formulations of the compositions including dosage units which may be incorporated into a variety of containers. The subject methods of administration generally involve contacting the cell with or administering to the host an effective amount of the subject compounds or pharmaceutically acceptable compositions. The compositions and compounds of the invention and the pharmaceutically acceptable salts thereof can be administered to a host in any effective way such as via oral, parenteral or topical routes. Preferred inhibitors are orally active in mammalian hosts.

In one embodiment, the invention provides the subject compounds combined with a pharmaceutically acceptable excipient such as sterile saline or other medium, gelatin, an oil, etc. to form pharmaceutically acceptable compositions. The compositions and/or compounds may be administered alone or in combination with any convenient carrier, diluent, etc. and such administration may be provided in single or multiple dosages. Useful carriers include solid, semi-solid or liquid media including water and non-toxic organic solvents. In another embodiment, the invention provides the subject compounds in the form of a pro-drug, which can be metabolically converted to the subject compound by the recipient host. A wide variety of pro-drug formulations are known in the art. The compositions may be provided in any convenient form including tablets, capsules, lozenges, troches, hard candies, powders, sprays, creams, suppositories, etc. As such the compositions, in pharmaceutically acceptable dosage

units or in bulk, may be incorporated into a wide variety of containers. For example, dosage units may be included in a variety of containers including capsules, pills, etc.

The compositions may be advantageously combined and/or used in combination with other therapeutic or prophylactic agents, different from the subject compounds. In many instances, administration in conjunction with the subject compositions enhances the efficacy of such agents, see e.g. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., 1996, McGraw-Hill. For diagnostic uses, the inhibitors or other KUZ binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

According to the invention, a KUZ protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which recognize such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to human KUZ are produced. In another embodiment, antibodies to the extracellular domain of KUZ are produced. In another embodiment, antibodies to the intracellular domain of KUZ are produced.

Various procedures known in the art may be used for the production of polyclonal antibodies to a KUZ protein or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of the KUZ protein encoded by a sequence selected from SEQ ID NOS: 1, 3, 5, 7 or 9 or a subsequence thereof, can be obtained. For the production of antibody, varioius host animals can be immunized by injection with the native KUZ protein, or a synthetic version, or derivative (*e.g.*, fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward a KUZ protein sequence or analog thereof, any technique which provides for the production of antibody molecules by

continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256: 495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80: 2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for KUZ together with genes from a human antibody molecule of appropriate biological acitvity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce KUZ-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for KUZ proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a KUZ protein, one may assay generated hybridomas for a product which binds to a KUZ fragment

containing such domain. For selection of an antibody immunospecific to human KUZ, one can select on the basis of positive binding to human KUZ and a lack of binding to a KUZ of another species. The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the protein sequences of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc. Antibodies specific to a domain of a KUZ protein are also provided. In a specific embodiment, antibodies which bind to a Notch-binding fragment of KUZ are provided.

The amino acid sequences of the disclosed KUZ polypeptides are used to back-translate KUZ polypeptide-encoding nucleic acids optimized for selected expression systems (Holler et al. (1993) Gene 136, 323-328; Martin et al. (1995) Gene 154, 150-166) or used to generate degenerate oligonucleotide primers and probes for use in the isolation of natural KUZ-encoding nucleic acid sequences ("GCG" software, Genetics Computer Group, Inc, Madison WI). KUZ-encoding nucleic acids used in KUZ-expression vectors and incorporated into recombinant host cells, e.g. for expression and screening, transgenic animals, e.g. for functional studies such as the efficacy of candidate drugs for disease associated with KUZ-modulated cell function, etc.

The invention also provides nucleic acid hybridization probes and replication / amplification primers having a KUZ cDNA specific sequence comprising SEQ ID NO:1, 3, 5, 7 or 9, and sufficient to effect specific hybridization thereto (i.e. specifically hybridize with SEQ ID NO:1, 3, 5, 7 or 9, respectively, in the presence of an embryonic cDNA library from the corresponding species, and preferably in the presence of BMP cDNA as described by Howard and Glynn (1995). Such primers or probes are at least 12, preferably at least 24, more preferably at least 36 and most preferably at least 96 bases in length. Demonstrating specific hybridization generally requires stringent conditions, i.e. those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium titrate/0.1% SDS at 50°C., or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium

pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 (g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C., with washes at 42°C. in 0.2 x SSC and 0.1% SDS. KUZ nucleic acids can also be distinguished using alignment algorithms, such as BLASTX (Altschul *et al.* (1990) Basic Local Alignment Search Tool, J Mol Biol 215, 403-410).

The subject nucleic acids are of synthetic/non-natural sequences and/or are isolated, i.e. unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, preferably at least about 5% by weight of total nucleic acid present in a given fraction, and usually recombinant, meaning they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome. Recombinant nucleic acids comprising the nucleotide sequence of SEQ ID NO:1, 3, 5, 7 or 9, or the subject fragments thereof, contain such sequence or fragment at a terminus, immediately flanked by (i.e. contiguous with) a sequence other than that which it is joined to on a natural chromosome, or flanked by a native flanking region fewer than 10 kb, preferably fewer than 2 kb, which is at a terminus or is immediately flanked by a sequence other than that which it is joined to on a natural chromosome. While the nucleic acids are usually RNA or DNA, it is often advantageous to use nucleic acids comprising other bases or nucleotide analogs to provide modified stability, etc.

The subject nucleic acids find a wide variety of applications including use as translatable transcripts, knock-in/out vectors, hybridization probes, PCR primers, diagnostic nucleic acids, etc.; use in detecting the presence of KUZ genes and gene transcripts and in detecting or amplifying nucleic acids encoding additional KUZ homologs and structural analogs. In diagnosis, KUZ hybridization probes find use in identifying wild-type and mutant KUZ alleles in clinical and laboratory samples. Mutant alleles are used to generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. In therapy, therapeutic KUZ nucleic acids are used to modulate cellular expression or intracellular concentration or availability of active KUZ.

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a KUZ modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate KUZ interaction with a natural KUZ binding target such as a Notch protein, etc. A wide variety of assays for

binding agents are provided including labeled *in vitro* protein-protein binding assays including protease assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

Exemplary *in vitro* binding assays employ a mixture of components including an KUZ polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular KUZ binding target. In a particular embodiment, the binding target is a Notch protein-derived substrate of KUZ protease activity. Such substrates comprise a specifically KUZ-cleavable peptide bond and at least 5, preferably at least 10, and more preferably at least 20 naturally occurring immediately flanking residues on each side. While native full-length binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject KUZ polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like ATP or ATP analogs (for protease assays), salts, buffers, neutral proteins, e.g. albumin, detergents, non-specific protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the KUZ polypeptide specifically binds the cellular binding target, portion or analog with a reference binding affinity. The mixture components can be added in any order that provides for the requisite bindings and incubations may be performed at any temperature which facilitates optimal binding. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening.

After incubation, the agent-biased binding between the KUZ polypeptide and one or more binding targets is detected by any convenient way. For KUZ protease assays, 'binding'

is generally detected by the generation of a KUZ substrate cleavage product. In this embodiment, protease activity may quantified by the apparent transfer a label from the substrate to the nascent smaller cleavage product, where the label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

A difference in the binding affinity of the KUZ polypeptide to the target in the absence of the agent as compared with the binding affinity in the presence of the agent indicates that the agent modulates the binding of the KUZ polypeptide to the KUZ binding target. Analogously, in cell-based assays described below, a difference in KUZ-dependent modulation of signal transduction in the presence and absence of an agent indicates the agent modulates KUZ function. A difference, as used herein, is statistically significant and preferably represents at least a 50%, more preferably at least a 90% difference.

Altered *Drosophila* hosts in which the *kuz* gene is over-expressed, under-expressed, mis-expressed or expressed as a variant are used to identify compounds that are antagonist or agonists of the KUZ polypeptide as well as to identify genes that encode products that interact with the KUZ polypeptide using art known methods (Xu et al., Genes and Devel., p464-475 (1990), Simon *et al.*, Cell, 67:701-716 (1991) and Fortini et al., Cell, 79:273-282 (1994)).

Agents that modulate the interactions of the KUZ polypeptide with its ligands/natural binding targets can be used to modulate biological processes associated KUZ function, e.g. by contacting a cell comprising a KUZ polypeptide (e.g. administering to a subject comprising such a cell) with such an agent. Biological processes mediated by KUZ polypeptides include a wide variety of cellular events which are mediated when a KUZ polypeptide binds a ligand e.g. cell differentiation, cell development and neuronal partitioning. The agents are also used to modulate processes effected by KUZ substrates; for example, *Notch*, an art known peptide involved in neurogenesis is over-expressed in some forms of leukemia (Ellison et al., Cell, 66:649-661 (1991)).

The present invention further provides methods for identifying cells involved in KUZ polypeptide-mediated activity, e.g. by determining whether the KUZ polypeptide, or a *kuz* ligand, is expressed in a cell. Such methods are useful in identifying cells and events involved in neurogenesis. In one example, an extract of cells is prepared and assayed by of a variety of immunological and nucleic acid techniques to determine whether the KUZ polypeptide is expressed. The presence of the KUZ polypeptide provides a measurement of the participation or degree of neurogenesis of a cell.

The invention provides a wide variety of methods and compositions for evaluating modulators of the KUZ signaling pathways. For example, the invention provides transgenic non-human animals such as flies (e.g. *Drosophila*), worms (e.g. *C. elegans*), mice, etc. having at least one structurally and functionally disrupted KUZ allele. In particular embodiments, the animals comprise a transgene within a KUZ allele locus, encoding a selectable marker and displacing at least one exon of the KUZ allele. More particularly, the transgene may comprise 3' and 5' regions with sufficient complementarity to the natural KUZ allele at the locus to effect homologous recombination of the transgene with the KUZ allele. Such animals provide useful models for determining the effect of candidate drugs on a host deficient in KUZ function.

As describe above, the invention provides a wide variety of methods for making and using the subject compositions. As additional examples, the invention provides methods for determining the effect of a candidate drug on a host deficient in KUZ function, such as: contacting a transgenic animal having at least one disrupted KUZ allele with a candidate drug; and, detecting the presence or absence of a physiological change in the animal in response to the contacting step. The invention also provides methods of evaluating the side effects of a KUZ function inhibitor, such as: contacting a transgenic animal having at least one disrupted KUZ allele with a candidate drug; detecting the presence or absence of a physiological change in the animal in response to the contacting step, wherein the presence of a physiological change indicates the inhibitor has side effects beyond KUZ function inhibition.

Without further description, one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples

therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure. Other generic configurations will be apparent to one skilled in the art. All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

EXAMPLES

Example 1: Identification of a Drosophila KUZ polypeptide/gene

Genes involved in lateral inhibition were screened using FLP/FRT chromosomes to produce mutant clones in mosaic animals (T. Xu and G.M. Rubin, *Development* 117:1223 (1993); T. Xu and S. Harrison *Methods in Cell Biology* 44:655 (1994)) and to isolate several alleles of a gene family designated herein as *kuzbanian (kuz)*. The *kuz* locus is defined by a single complementation group which maps to chromosomal location 34C4,5, and corresponds to the 1(2)34 Da group (A.C. Spradling *et al., PNAS* 92:10824 (1995). Most of the *kuz* phenotypic analysis was performed using the null allele *kuze29-4*. *Kuze29-4* is an excision allele deleting approximately 2.4 kb at the 5' end of the *kuz* gene, including DNA in the promoter region and the first and second exons. Four *P[lacZ; w+]* insertions 1(2)k11804, 1(2)k01403, 1(2)k07601 and 1(2)k14701 are hypomorphic *kuz* alleles. These insert either in the first *kuz* exon or in the first intron. Precise excision of these P insertions reverts the associated *kuz* phenotype. *Kuz1* is the original *kuz* allele caused by an insertion of 4.3 kb of DNA in or near the first exon. Seventeen additional X-ray induced *kuz* alleles were isolated in the FLP/FRT mosaic screen.

A 10 kb fragment of DNA from the region deleted in allele *kuze29-4* was used to screen a *Drosophila* total imaginal disc cDNA library. A group of two overlapping 1.2 kb cDNAs mapping to this region was recovered; a full-length *kuz* cDNA, NB1, was isolated from an embryonic cDNA library using the small cDNA clones as probes (*Kuz* cDNA Genbank accession number: U60591).

Scanning electron microscopy (SEM) and embryo staining and adult eye sections were carried out following standard procedures (A. Tomlinson and D.F. Ready, *Dev. Biol.* 123:264 (1987); T. Xu and S. Artavanis-Tsakonas, *Genetics* 126, 665 (1990)). A scanning

electron micrograph (SEM) showing the multiple bristle phenotype in an adult mosaic fly with homozygous *kuz* clones revealed that aeveral macro- and microchaete positions have supernumerary bristles whereas others are missing in the same area. SEMs showing *kuz* clones in the eye revealed the regular array of ommatidia is severely disrupted, that toward the center of the clone the density of photoreceptors is abnormally low and none are successfully organized into ommatidia, and that chimeric ommatidia at the clone border contain a mixture of pigmented wild-type photoreceptor cells and mutant, unpigmented photoreceptors. Confocal images of embryos stained with the neuronal-specific anti-Elav antibody demonstrate a requirement for maternal and zygotic *kuz* products. A *kuz* maternal null embryo (generated using the *ovoD* mutation as described in T.B. Chou and N. Perrimon, *Genetics* 131:643 (1992)) with one zygotic copy of *kuz* revealed that a greater proportion of the embryo developed as neural tissue than in wild-type and a surface view of a *kuz* null embryo with no maternal or zygotic *kuz* product showed that most cells adopted a neural fate. A lower focal plane of this same embryo showed that all cells around the periphery of the embryo are neural cells. A cuticular preparation of a *kuz* maternal null embryo with one zygotic copy of *kuz* showed a small patch of cuticle develops on the dorsal side of the embryo; presumably the remaining cells which failed to produce cuticle adopted a neural fate, consistent with the previously phenotype. A cuticular preparation of a *kuz* null embryo showed only a tiny dot of cuticle developed. Most of these embryos show no cuticle at all.

Animals with *kuz* mutant clones exhibit clusters of sensory bristles at positions in which single sensory bristles are normally observed. Separate sockets are often seen with individual bristles, and stimulation of mutant bristles in a reflex test elicits a leg cleaning response, indicating that mutant clusters contain multiple sensory bristles and not just multiple shafts (P. Vandervorst and A. Ghysen, Nature 286:65 (1980)). This multiple bristle phenotype is observed in clones mutant for several neurogenic genes such as *Notch (N)* and *shaggy (sgg*, also known as *zeste-white 3*), and is indicative of a failure of lateral inhibition during the development of the peripheral nervous system (S. Artavanis-Tsakonas, *et al.*, *Trends in Genetics* 7:403 (1991); J.S. Campos-Ortega (1993); Jan, Y.N. and Jan, L.Y., *id.*, pp. 1207-1244; Romani, S. *et al.*, *Genes Dev.* 3:997 (1989); Artavanis-Tsakonas, S. *et al.*, *Science* 268:225 (1995); Heitzler, P. and Simpson, P. (1991). Cell *64*, 1083-1092).

Unlike the *N* phenotype, *kuz* clones do not produce ectopic bristles, indicating *kuz* is not required for correct spacing between proneural clusters. Mutant clones in the adult eye severely disrupted the regular array of ommatidia. Thin sections through such a mosaic eye reveal that mutant photoreceptors are not organized correctly into ommatidia.

To determine whether the KUZ polypeptide is required for the development of the central nervous system (CNS), embryos lacking any maternally derived KUZ polypeptide and containing one or no zygotic copies of the *kuz* gene were produced. The embryos were examined by staining with neuronal-specific antibodies to the Elav protein (Bier, E. *et al.*, *Science* 240:913 (1988); Robinow, S. *et al.*, *J. Neurobiol.* 22, 443 (1991)). Maternal null embryos with one copy of zygotic *kuz* gene showed hyperplasia and disorganization of the CNS on the ventral side of the embryos, which is a phenotype similar to the neurogenic phenotype of *N* mutant embryos (Lehmann, R. *et al.*, *Roux's Arch Dev. Biol.* 192:62 (1983)). However, embryos lacking all maternal and zygotic KUZ polypeptide have a much more severe neurogenic phenotype. Hypertrophy of the nervous system is not restricted to the ventral region, but the embryos stained throughout with anti-Elav, demonstrating that many more cells in the embryo had developed as neural cells. Such a severe neuralizing phenotype is similar to that of *sgg* null embryos (Bourouis, M. *et al., Nature* 341:442 (1989)). Cuticular preparation of embryos correlated well with the antibody results: Maternal-null embryos with one copy of the *kuz* gene produced a small patch of cuticle on the dorsal side, consistent with the observation that many of the ventral cells had adopted a neural fate at the expense of epidermis. Embryos with no KUZ polypeptide produced little or no cuticle, as would be expected if most cells had become neural, leaving few epidermal cells to secrete cuticle.

Further analyses on the development of adult sensory bristles were performed to determine a specific role for the KUZ polypeptide in lateral inhibition. The *yellow (y)* and *crinkle (ck)* marker mutations were used to mark *kuz*- clones in the adult cuticle. This allows one to determine the genotype of individual cells and thus to examine the autonomy of the *kuz* mutant phenotype. Such analysis can distinguish between sending and receiving roles for a gene involved in the lateral inhibition process (Heitzler, P. *et al.*, *Cell* 64:1083 (1991)).

A role for the KUZ polypeptide in lateral inhibition is suggested by the observation that all sensory bristles in a mutant cluster are *kuz*-; no wild-type bristles are ever present in a cluster. SEM of *kuz*- clones (each *kuz*- cell is also *ck*- and *y*-) revealed that the *ck*- mutation

results in extra trichomes in the epidermal cell and in blunted shafts of sensory bristles; these morphological changes allow the border between mutant and wild-type cells to be precisely determined. A marked absence of all micro- and macrochaetes is observed in the interior of the clone, as no shafts, sockets, or neurons (naked cells) are seen. *Kuz-* mutant cells at normal bristle positions do form bristles at clone borders where they are in contact with wild-type cells. A high- magnification view of one of the multiple macrochaete clusters at a clone border revealed that every bristle in this and other clusters is always *ck-* and *y-*, demonstrating that all bristles in a cluster are *kuz-*. No wild-type bristles are observed in multiple bristle clusters. Marked *kuz-* clones were generated in *y- w- hsFLP1; kuse29-4 ck-P[FRT]40A/P[y+] P[w+]P[FRT]40A* first instar larvae following protocols described in T. Xu and G.M. Rubin, *Development* 117:1223 (1993) and T. Xu and S. Harrison *Methods in Cell Biology* 44:655 (1994).

Mosaic analysis for *kuz-* clones in the adult cuticle indicates two distinct functions for the *kuz* protein. First, the failure of lateral inhibition, evidenced by the formation of extra bristles, only occurs in *kuz-* mutant cells. This cell-autonomous mutant phenotype indicates that during normal development, the kuz protein is required in cells to receive an inhibitory signal. *kuz-* cells at normal bristle-forming positions become bristles only when they are in contact with wild-type cells, indicating that in wild-type animals, the KUZ polypeptide may act as a positive signal or is involved in sending a positive signal for the development of the bristle. Thus, there is a cell-autonomous requirement for *kuz* in order for cells to be inhibited from adopting a neural precursor fate. We conclude that the KUZ polypeptide is required in cells to receive an inhibitory signal from the emerging neural cell. Cells in the proneural cluster with wild-type KUZ polypeptide function receive the inhibitory signal and are forced to become epidermal, whereas *kuz-* cells cannot be inhibited and develop as neural precursor cells. A second distinct role for the KUZ polypeptide was revealed by the same mosaic analyses. All mutant bristle clusters were produced at clone borders, where mutant cells contact wild-type cells. No bristles were ever produced in clone interiors, either singly or in clusters. Large *kuz-* clones therefore cause bare patches devoid of bristles containing only hair-secreting epidermal cells. This phenotype indicates there is a non cell-autonomous requirement for the KUZ polypeptide in bristle development. Hence, *Kuz* participates in both neural-promoting and -inhibiting processes during formation of the adult epidermis.

To reveal the molecular basis of the KUZ polypeptide functions, a *kuz* gene was cloned and a full-length cDNA was obtained. The *kuz* cDNA contained an open reading frame that encodes a1,239 amino acid membrane-spanning protein of the metalloprotease-disintegrin family known as the ADAM family (members of the ADAM family contain "A Disintegrin And Metalloprotease Domain". The *KUZ* metalloprotease domain also contains a conserved zinc-binding site (Jiang, W. and Bond, J. S. (1992). FEBS Letters *312*, 110-114). Like other disintegrins KUZ has a characteristic spacing of cysteine residues that is required for their direct binding to receptors (Niewiarowski, S. *et al.*, *Seminars in Hematology* 31:289 (1994)). These cysteines are conserved in the KUZ polypeptide along with many additional residues that are shared by other disintegrin domains. In this family, many proteins with a multi-domain structure are proteolytically processed to produce multiple peptides with different functions (Blobel, C.P. *et al.*, *J. Cell Biol.* 111:69 (1990); Neeper, M.P. *et al.*, *Nucleic Acids Res.* 18:4255 (1990); Au, L.C., *et al.*, *Biochem. Biophys. Res. Commun.* 181:585 (1991)). The metalloprotease and disintegrin domains of *kuz* may be cleaved from the full-length precursor to produce both soluble and membrane-bound forms of these domains. Such proteolytic products of the KUZ polypeptide may be used to carry out the different KUZ polypeptide functions.

<u>Example 2: Identification of two human and one mouse KUZ polypeptides/genes.</u>

The nucleic acid sequence of the *Drosophila kuz* gene was used to generate PCR primers for amplifying *kuz* encoding nucleic acid molecules from organisms other than *Drosophila*. A .9kb cDNA fragment was amplified from a human fetal brain cDNA library (Clonetech, Stratagene) using PCR primers. This fragment was cloned and was used as a probe to screen the human fetal brain cDNA library (Clonetech, Stratagene). A clone containing a 3.5kb insert was obtained (SEQ ID NO:3). The cloned contained a full length encoding sequence that encodes a protein of 749 amino acids. Three additional clones were obtained that showed variant restriction digestion patterns. Sequence analysis of these clones identified a second form of the human KUZ polypeptide. This second form of the KUZ polypeptide encodes a protein of 330 amino acids in length (SEQ ID NO:6). A fragment of the human *kuz* encoding sequence was used to probe a mouse fetal brain cDNA library. One of four isolated clones was sequenced and contained a 4kb insert representing a murine KUZ cDNA (SEQ ID NO:7).

Northern blots run using RNA isolated from various mouse and human tissues revealed expression in fetal and adult tissues. Hybridization of the blots with probes specific to each of the human forms confirmed that each of the transcripts was unique to one of the two forms, indicating that the two identified mRNA transcripts represent each of the two human forms respectively. The variable pattern of expression seen on the adult and fetal Northern blots indicates a developmental role of the KUZ polypeptides: the short form being predominant in adult tissues while the full length form is predominant in fetal tissues and adult brain. All regions of the adult brain expressed both forms.

Example 3: KUZBANIAN controls proteolytic processing of NOTCH and mediates lateral inhibition during Drosophila and vertebrate neurogenesis.

To investigate how the different domains of KUZ contribute to its biological functions, full length and various N- and C- terminal truncations of KUZ were generated (e.g. constructs 1-4 and 7, Fig. 1B) and expressed under the pGMR vector (Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Development *120*, 2121-2129) in the developing retina of Drosophila. One of these exemplary truncations (7), which is missing the protease domain, resulted in a dominant rough eye phenotype. We expressed KUZ truncations using the pDMR vector which contains the *decapentaplegic* (*dpp*) disc specific enhancer element (see experimental procedures) that drives gene expression in several tissues including parts of the notum and the wing blade, two tissues that are known to be affected in *kuz* mutant clones. Expression of construct 7 under pDMR resulted in supernumerary bristles on the notums and notches on the wing blades. These phenotypes resemble those seen in somatic clones homozygous for *kuz* loss-of-function mutations, indicating that this construct functions in a dominant negative manner by interfering with endogenous *kuz* activity. We also observed that the mutant phenotypes resulting from this construct are dominantly enhanced by removing one copy of the endogenous *kuz* gene; that is, the phenotypes of *kuz*/+ individuals carrying this construct are more severe than those of +/+ individuals. Conversely, additional wildtype KUZ protein from a transgene expressing full length KUZ suppresses these phenotypes. We refer to the particular dominant negative of construct 7 hereafter as KUZDN (KUZ dominant negative).

To directly address the functional relevance of the protease domain, we introduced into full length KUZ a point mutation (E606 to A) in the putative zinc binding site (Fig. 1A)

of the protease domain. This glutamate is thought to be a catalytic residue and is absolutely conserved among all known metalloproteases (Jiang and Bond, 1992). Thus, this point mutation should abolish protease activity while having minimal impact on the other activities of KUZ. Indeed, overexpression of KUZ^{E606A} (construct 8 in Fig. 1B) gave similar, although somewhat weaker, dominant phenotypes to those seen with KUZDN.

The notums of Drosophila adults carry two types of sensory bristles, macrochaetes and microchaetes. The sensory organ precursor cells (SOPs) that generate the macrochaetes are selected from pools of equivalent cells by lateral inhibition mostly during the third instar larval stage, while the SOPs for the microchaetes are singled out during the early pupae stage (Huang, F., et al. (1991). Devélopment *111*, 1087-1095; Hartenstein, V. and Posakony, J. W. (1989). Development *107*, 389-405). *N* is required for this process and removal of *N* function at larval and pupal stages differentially affects these two types of bristles (Hartenstein, V. and Posakony, J. W. (1990). Dev. Biol. *142*, 13-30). If KUZ is required for lateral inhibition, we would expect to generate similar phenotypes by expressing KUZDN at these times. We generated flies containing KUZDN under the control of the *hsp70* promoter, and applied one hour heat pulses at various times during larval and pupal development. We observed that while heat pulses applied during third instar larval stage resulted in supernumerary macrochaetes only, heat pulses applied during early pupal stages (0-13 hrs after puparium formation (APF)) resulted in supernumerary microchaetes only, similar to the phenotypes resulted from removing *N* function at these times using a temperature sensitive *N* allele (Hartenstein and Posakony, 1990). These time points match the periods when SOPs for each bristle type are selected from pools of equivalent cells (Huang et al., 1991; Hartenstein and Posakony, 1989), indicating that KUZDN interferes with lateral inhibition during the selection of SOPs.

Kuz mutant clones affect other tissues such as the eye. We perturbed *kuz* functions by expressing KUZDN under the control of the *rough* enhancer, which drives gene expression in all cells within the morphogenetic furrow as well as transiently in R2, R5, R3 and R4 posterior to the furrow (Heberlein, U., et al. (1994). Mech. Dev. *48*, 35-49). Flies carrying the *rough*/KUZDN transgene had supernumerary photoreceptor cells in each ommatidium. Neuronal differentiation in these transgenic flies was followed by staining for ELAV, a neuronal marker, in eye imaginal discs. Consistent with the adult eye phenotype, we observed

the recruitment of extra neurons into each ommatidial cluster in the developing retina. These experiments indicate that *kuz* function is required to limit the number of photoreceptor neurons recruited into each ommatidium.

Besides its functions in determining neural fate, *kuz* is also required for axonal extension at later stages of neural development (Fambrough, D., et al. (1996). Proc. Natl. Acad. Sci. USA *93*, 13233-13238). We expressed KUZDN under the control of the ELAV promoter using the GAL4-UAS system (Brand, A. H., and Perrimon, N. (1993). Development *118*, 401-415). The ELAV promoter drives gene expression in maturing and mature neurons, but not neuroblasts, thus allowing one to bypass the requirement for *kuz* in neural fate determination. We observed that embryos expressing KUZDN in developing neurons show major defects in axonal pathways, such as disruption of longitudinal axonal tracts. In general, this phenotype is similar to the that observed in zygotic *kuz* mutant embryos (Fambrough et al., 1996), indicating that KUZ provides a proteolytic activity synthesized by axons and required by them to extend growth cones through the extracellular matrix.

Database searches revealed sequences representing putative *kuz* orthologs in C. elegans, rat, bovine and human. The bovine homolog was initially isolated as a proteolytic activity on myelin basic protein *in vitro* (Howard et al., 1996). We isolated and sequenced cDNAs representing a full-length mouse *kuz* homolog. This mouse protein (MKUZ) is 45% identical in primary sequence with Drosophila KUZ (DKUZ, Fig. 1), and 95% identical with the bovine protein. Sequence similarity between MKUZ and DKUZ extends over the whole coding region, except that MKUZ, like other vertebrate KUZ homologs, has a much shorter intracellular domain. The intracellular domain of MKUZ contains a stretch of 9 amino acid residues (934-942) that are absolutely conserved with DKUZ. To determine the functional importance of this sequence similarity, we introduced into KUZDN mutations in several conserved residues in this region (936TPSS939 to AAAA; construct 9 in Fig. 1B) and found these mutations dramatically reduced KUZDN activity.

Based on the structure of KUZDN described above, we engineered a dominant negative form of MKUZ (MKUZDN, Fig. 1B) missing the protease domain. When overexpressed in Drosophila using the pDMR vector, MKUZDN resulted in dominant phenotypes resembling those created by its Drosophila counterpart. To test directly the involvement of MKUZ in vertebrate neurogenesis, we injected *in vitro* transcribed mRNA

encoding MKUZDN into Xenopus embryos. Primary neurons in Xenopus are generated in precise and simple patterns and can be identified by their expression of a neural specific *β-tubulin* gene (*N-tubulin*). This assay has been used previously to demonstrate a conserved role for certain neurogenic genes in singling out primary neurons in Xenopus by lateral inhibition (Chitnis, A., et al. (1995). Nature *375*, 761-766). If a *kuz*-like activity is required for the lateral inhibition process in Xenopus, we would expect interference with this endogenous *kuz* activity to result in the overproduction of primary neurons. Indeed, injection of mRNA encoding MKUZDN resulted in extra *N-tubulin* positive cells. Consistent with the notion that *kuz* acts to limit the number of cells that differentiate as neurons from a group of competent cells, these extra *N-tubulin* positive cells were confined to domains of primary neurogenesis, and were not observed at ectopic positions.

To provide further evidence for an endogenous *kuz* activity during primary neurogenesis in Xenopus, we examined the expression pattern of a Xenopus *kuz* homolog (*Xkuz*). A cDNA fragment representing a portion of *Xkuz* (Fig. 1) was isolated (see experimental procedures) and used to generate RNA probes for *in situ* hybridization under high stringency. *Xkuz* is expressed uniformly in gastrulating and neural plate stage embryos, including the domains of primary neurogenesis. In older embryos, *Xkuz* continues to be widely expressed, with an elevated level in neural tissues. Thus, *Xkuz* is expressed at the appropriate time and place for a potential role in primary neurogenesis in Xenopus.

We sought to determine the order of action of *N* and *kuz* by examining the phenotype produced by combining a gain-of-function *N* mutant and a loss-of-function *kuz* mutant. Expression of an activated form of NOTCH (reviewed in Artavanis-Tsakonas et al., 1995) under the heat shock promoter (hs-N^{act}) at early pupal stages (7-9 hours APF) leads to the loss of microchaetes on the notum; the opposite phenotype, extra microchaetes, is seen in loss-of-function *kuz* mutant clones. We focused on microchaetes since the SOPs for these bristles are generated more synchronously than those of the macrochaetes (Huang et al., 1991; Hartenstein and Posakony, 1989) and thus a single pulse of heatshock at 7-9 hrs APF results in the reproducible loss of microchaetes on the notum in hs-N^{act} flies. If *kuz* acts genetically downstream of *N*, then the combination of N^{act} and *kuz* should display the *kuz* phenotype of extra microchaetes. Conversely, if *kuz* acts genetically upstream of *N*, then the combination of N^{act} and *kuz* should display the N^{act} phenotype of missing microchaetes. We observed that

the combination of N^{act} and *kuz* displayed the N^{act} phenotype, indicateing that *kuz* acts genetically upstream of *N*. This result indicates KUZ acts upstream of, or parallel with NOTCH in the same biochemical pathway.

We observed dosage sensitive genetic interactions between *kuz* and *N*, indicating that the levels of activity of *kuz* and *N* are tightly balanced. We took advantage of a weak *dpp*-KUZDN transgene that resulted in an average of 3 posterior scutellar bristles instead of the 2 seen in wildtype. While heterozygous *N* mutants have normal number of posterior scutellar bristles, this genetic background dramatically enhanced the phenotype resulting from the weak *dpp*-KUZDN transgene such that an average of 5.2 bristles (n=50) were observed. Furthermore, in flies that carry an additional copy of *N* gene, the extra bristle phenotype resulting from this KUZDN transgene is completely suppressed such that 2 bristles were observed. This intricate balance between their activities indicates that *kuz* and *N* are closely linked in a common biological process.

We examined if perturbation of KUZ function in Drosophila Schneider 2 (S2) cell cultures would affect NOTCH processing. S2 cells do not express any endogenous NOTCH protein (Fehon et al., 1990), but do express high levels of *kuz* mRNA. Upon transfection of a full-length *N* construct, the monoclonal antibody C17.9C6, which was raised against the intracellular domain of NOTCH, can detect full length NOTCH (about 300 kd) and C-terminal fragments of about 100 kd (Fehon et al., 1990). We reasoned that if *kuz* is involved in generating this 100 kd species in S2 cells, then expression of KUZDN might interfere with this proteolytic event. Indeed, expression of KUZDN nearly abolished the 100 kd species in S2 cells, while the 300 kd species was not greatly affected, indicating that *kuz* is required for the NOTCH processing. Consistent with our results in transgenic flies that overexpression of full length KUZ did not perturb neurogenesis, transfection of a full length KUZ construct did not affect NOTCH processing in S2 cells.

Next, we performed similar experiments in developing imaginal discs. As described earlier, in transgenic flies containing KUZDN under the control of the heatshock promoter, one hour heatshock at the third instar larval stage resulted in extra bristles on the notum. The same heatshock regime also resulted in notches on the wing blade and extra photoreceptors in the eye. We followed the status of NOTCH processing in the wing and eye imaginal discs after the induction of KUZDN in these animals. As in transfected S2 cells, mAb C17.9C6

normally detects a 300 kd and a 100 kd NOTCH species in protein extracts of the third instar imaginal discs. After the induction of KUZDN by one hour heatshock, the 100 kd species gradually disappears; by 4 hours after induction, the 100 kd species is almost undetectable, while the 300 kd species has accumulated to a higher level. By 15 hrs after the heatshock, the 100 kd species is restored to wildtype levels presumably reflecting the decay of the KUZDN protein synthesized in response to the heatshock. The correlation between the reduction of the 100 kd species upon KUZDN expression and the resulting neurogenic phenotypes in imaginal tissues indicates the functional significance of the 100 kd NOTCH form detected *in vivo*.

Finally, we examined NOTCH processing in *kuz* null mutant embryos. Since *kuz* is known to have a maternal contribution (supra), we generated germline clones to obtain embryos lacking all KUZ function. We found that while mAb C17.9C6 detects a 300 kd and a 100 kd species in wildtype embryos, only the 300 kd species is detected in *kuz* null embryos. This observation indicates that the phenotypes we generated by expression of KUZDN are not due to interference with genes other than *kuz*, such as other members of the ADAM family, and that *kuz* is required for the proteolytic processing of NOTCH (Fig. 2).

Our studies provide a general scheme for engineering dominant negative forms of ADAM proteins applicable to other ADAM genes. While all ADAMs possess a disintegrin-like and a metalloprotease-like domain, some ADAMs lack a consensus active site in the metalloprotease domain. These "protease dead" ADAMs resemble dominant negative forms of KUZ described herein and can function as endogenous inhibitors.

Experimental Procedures: Plasmid Constructs: We initially used the pGMR vector (Hay et al., 1994) to express full length KUZ and several N- and C- terminal deletion constructs in the eye. These constructs include 1, 2, 3, 4 and 7. Upon identification of 7 as a dominant negative form (KUZDN), we then used another expression vector pDMR to express constructs 1, 4, 5, 6, 7, 8 and 9. The pDMR vector utilizes the *dpp* disc specific enhancer to drive gene expression in multiple tissues including the wing and the notum. pDMR was constructed by the following steps. First, the heat shock responsive element in Casperhs (Pirotta, V. (1988). In Vectors: A Survey of Molecular Cloning Vectors and their Uses) was removed to yield Casperhs-1. A 4.3 kb *dpp* disc specific enhancer (Staehling-Hampton, K., et al.(1994). Cell Growth Differ. *5*, 585-593) was inserted upstream of the *hsp70* basal promoter in Casperhs-1 to yield pDMR (*dpp* mediated reporter). Construct 7 (KUZDN) was also

cloned into pUAST (Brand and Perrimon, 1993) and pCasperhs to generate UAS/KUZDN and hs/KUZDN, respectively. A *rough* enhancer element (Heberlein et al., 1994) was then inserted into hs/KUZDN to generate *rough*/KUZDN. Constructs 1 (full length KUZ) and 7 (KUZDN) were also cloned downstream of the metallothionein promoter in pRMHa-3, a S2 cell expression vector (Bunch, T. A., et al. (1988) Nucl. Acids Res. 16, 1043-1061). The nucleotide coordinates of constructs 1 through 9 are as follows, using the same numbering as in GenBank accession no. U60591. 1 and 8: 723-5630; 2: 723-3578; 3: 723-3462; 4: 723-2757; 5: 1957-2757; 6: 1957-5630; 7 and 9: 2757-5630. Note that for all the N- terminal deletion constructs, a DNA fragment (nucleotides 723-940) containing the signal peptide was provided at the 5' end. Site directed mutagenesis was carried out using Stratagene's QuickChange system.

MKUZDN was generated by an N- terminal truncation that removes the pro and catalytic domains of MKUZ. The rest of MKUZ (nucleotide 1483-2573) was ligated either to a DNA fragment (723-940, according to nucleotide coordinates in U60591) containing the signal peptide of Drosophila KUZ to generate MKUZDN-1 or to a fragment (nucleotide 1-248) containing the signal peptide of MKUZ to generate MKUZDN-2. MKUZDN-1 was subcloned into pDMR and pUAST for overexpression in Drosophila, and MKUZDN-2 was subcloned into a modified CS2+ vector (Turner, D. L. and Weintraub, H. (1994). Genes Dev. *8*, 1434-1447.) for RNA injection in Xenopus embryos (see below).

Characterization of *kuz* Homologs from Mouse and Xenopus: PCR primers corresponding to sequences of a rat gene similar to *kuz* (GenBank accession: Z48444) were used to amplify a fragment from a mouse brain cDNA library. PCR product was then used to screen oligo(dT) and random primed cDNA libraries from the mouse PCC4 cell line (Stratagene). Two overlapping cDNA, mkuz2 and mkuz3 were characterized and sequenced, which together comprised the whole coding region. mkuz 2 extends from nucleotide 430 to 2573 and mkuz3 extends from 1 to 1345.

Xenopus *kuz* was cloned by PCR using degenerate primers (XK1) and (XK4) which correspond to Drosophila KUZ sequence HNFGSPHD and GYCDVF, respectively. First strand cDNA from stage 18 Xenopus embryos was used as template in a standard PCR reaction with an annealing temperature of 50°C. A PCR product of expected size was purified and used as template for another PCR reaction using a nested primer (XK3), corresponding to

Drosophila KUZ sequence EECDCG, and XK4. The PCR product was subcloned into Bluescript and sequenced. Anti-sense RNA was used as a probe for whole mount *in situ* hybridization of Xenopus embryos according to standard procedures (Harland, R. (1991). Meth. Cell Biol. *36*, 685-695).

For RNA injections in Xenopus embryos, MKUZDN-2 was synthesized *in vitro* using SP6 RNA polymerase from a CS2+ vector. Nuclear *lacZ* RNA was synthesized from plasmid pSP6nucßGal. 500 pg of MKUZDN RNA, together with 100 pg of *lacZ* RNA was injected into one blastomere of Xenopus embryos at 2-4 cell stage. *lacZ* RNA was also injected alone as a control. Embryos were fixed at the neural plate stage and stained with Red-Gal (Research Organics, Inc.). Embryos were then processed for *in situ* hybridization with a neural specific *β-tubulin* probe.

Drosophila Genetics: For epistasis between *kuz* and *Notch*, an activated *N* construct containing only the cytoplasmic domain of NOTCH (N^{act})under the control of the heatshock promoter (ITM3A insertion on the X chromosome, from Lieber, T., et al. (1993). Genes Dev. *7*, 1949-1965) and a null *kuz* allele *e29-4* (Rooke et al., 1996) were used. Flies of the genotype ITM3A/+; *e29-4 ck* FRT40A/+ were crossed to hsFlp/Y; FRT40A. The progeny from such a cross were subjected to a one hr heatshock at 38°C 24 to 48 hrs after egg laying to induce *kuz* mutant clones and another one hr heatshock at 7-9 hrs APF to induce the expression of N^{act}. Adult flies were processed for scanning electron microscopy and the clones identified by the cell autonomous *ck* epidermal hair marker as in Rooke et al. (1996).

kuz germline clones were generated as in Rooke et al. (1996). Females bearing germline clones were mated to *e29-4/CyO* males. *kuz* null embryos lacking both maternal and zygotic contribution can be distinguished from *kuz* maternal null embryos rescued with one zygotic copy of *kuz* at late embryonic stages since *kuz* null embryos fail to develop any cuticle while paternally rescued embryos develop some cuticle structures. *kuz* null embryos were hand-picked for making protein extracts.

Protein Extracts and Immunoblotting: About $2x10^6$ S2 cells, 50 embryos, or imaginal discs from 16 third instar larvae were used for each extraction. These materials were homogenized and incubated for 20 min on ice in 90 μl of buffer containing 10 mM KCl, 20 mM Tris pH 7.5, 0.1% mercaptoethanol, 1 mM EDTA plus protease and phosphatase inhibitors (leupeptin, aprotinin, PMSF and sodium vanadate). Supernatant was collected after

a low speed spin of 2000 rpm for 5 min. 12 µl of supernatant was run on a 6% SDS polyacrylamide gel. Blotting, antibody incubation, and chemiluminescent detection using the ECL kit were as described in Fehon et al. (1990).